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The *CTLA4 +49 A/G*G–CT60*G* haplotype is associated with susceptibility to multiple sclerosis in Flanders

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system white matter characterized by inflammation, demyelination and axonal damage. The cytotoxic T lymphocyte antigen-4 (CTLA-4) protein plays a key role in the down-regulation of T cell activation. We analysed the *CTLA4* +49A/G and CT60 polymorphisms in a cohort of 120 MS trio families recruited from the Flanders region in Belgium. Both polymorphisms were genotyped by polymerase chain reaction—restriction fragment length polymorphism (RFLP). The +49 G-allele was significantly more transmitted to affected probands ($P=0.005$). No transmission distortion was observed for the CT60 polymorphism. Haplotype analysis revealed significant overtransmission of the *+49 A/G*G–CT60*G* haplotype ($P=0.0025$), and undertransmission of the *+49 A/G*A–CT60*G* haplotype ($P=0.015$). The *CTLA4* gene has been the focus of intense investigation in MS. Of 15 recently published papers, only six reported significant associations of various *CTLA4* polymorphisms with MS, with the remainder being negative. Ours is the first report investigating the CT60 polymorphism in MS. Our data highlight a need for further scrutiny of the *CTLA4* gene in MS.

Keywords: CTLA-4; +49 A/G; CT60; Multiple sclerosis; Susceptibility; Association; Single nucleotide polymorphism

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system white matter resulting in extensive inflammation, demyelination and axonal damage (Compston and Coles, 2002; Paty and Ebers, 1998). In a vast majority of the patients, the disease consists of alternating recurrent attacks followed by a period of a variable degree of recovery (relapsing–remitting, RR), whereas in a minority of patients, the disease shows a more progressive course (primary progressive, PP). Both familial occurrence of MS and the higher concordance rate in monozygotic twins as compared to dizygotic twins point towards the importance of genetic factors (Ebers and Sadovnick, 1994). Following a series of genome-wide screens in MS, substantial evidence has emerged for the potential involvement of many genes, each contributing a small to moderate effect to the overall disease process (Dyment et al., 2004; Oksenberg et al., 2001).

Genes encoding proteins that play crucial roles in regulating the immune response, such as the cytotoxic T lymphocyte antigen 4 (CTLA-4), are considered to be of high relevance in autoimmune diseases such as MS. The CTLA-4 protein, the cell designation 28 antigen (CD28) and their ligands, B7-1 and B7-2, form the major pathway for the activation of T cells. While the CD28–ligand interaction brings about an increase in T cell response, the CTLA-4–ligand interaction has an inhibitory effect on T cell activation (Oosterwegel et al., 1999).

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The CTLA-4 gene has been mapped to chromosome 2q33.3 (Dariavach et al., 1988) and consists of 4 exons. The four most frequently studied polymorphisms are a dinucleotide repeat in the 3′ untranslated region, a G/A transition in exon 1 at position +49, a C/T transition in the -318 position of the promoter sequence and more recently a C/T transition (CT60) within the 3′-untranslated region (Ueda et al., 2003). The +49 A/G transition leads to an alanine to threonine amino acid substitution, with the G allele found to be associated with predisposition to many autoimmune diseases. The CT60 A allele has been shown to be protective while the G allele increases susceptibility to several autoimmune diseases (Ueda et al., 2003). In the latter study, the authors also showed that the G allele was associated with lower mRNA levels of soluble CTLA-4 isoform, thus providing a rationale for a functional role in susceptibility to autoimmune diseases.

Table 1 summarises all the studies that have been done to date investigating the effects of polymorphisms in the CTLA-4 gene in MS (see also Walsh et al., 2003 for a meta-analysis on the subject). Results have been conflicting with some studies (Malferrari et al., 2005; Alizadeh et al., 2003; Kantarci et al., 2003; Mäurer et al., 2002; Harbo et al., 1999; Ligers et al., 1999) hinting towards association with MS, while others (Fukazawa et al., 2005; Bonetti et al., 2004; Teutsch et al., 2004; Biliñska et al., 2004; van Veen et al., 2003; Bocko et al., 2003; Dyment et al., 2002; Masterman et al., 2002; Rasmussen et al., 2001) claiming to find no association at all. In view of this ambiguity, we decided to investigate the effect of 2 single nucleotide polymorphisms

Table 1
Published association studies on CTLA-4 in MS

Association	Ethnicity	Study size	Polymorphism(s) studied	Major findings
Positive				
Malferrari et al. (2005)	Italy	95 MS vs. 104 C	−318 C/T +49 A/G	*−318*T−+49*G* associated with MS (P=0.015)
Alizadeh et al. (2003)	France Italy Portugal	411 simplex families 113 simplex families 86 simplex families	−651 C/T −318 C/T HLA type	Association with *−651*C* allele in *HLA-DRB1*15* patients (P=0.0002 for French and P=0.02 for Italian and Portuguese)
Kantarci et al. (2003)	USA	122 MS vs. 244 C 59 multiplex families	−318 C/T +49 A/G $(AT)_n$	Association of homozygous haplotype *−318*C−+49*A−(AT)$_8$* (P<0.02). No linkage in families
Mäurer et al. (2002)	Germany	330 MS vs. 152 C	+49 A/G	No difference between MS and C; *+49*G* allele associated with PP vs. RR/SP (P=0.02)
Harbo et al. (1999)	Norway	296 MS vs. 271 C	−318 C/T +49 A/G	+49 A/G genotype associated with MS (P=0.01); RR phenotype vs. C (P=0.006)
Ligers et al. (1999)	Sweden	46 multiplex families 28 simplex families 237 C	−318 C/T +49 A/G $(AT)_n$	+49 G/G genotype associated with MS (P=0.0471); TDT for *+49*G* (P=0.02); Strong linkage for $(AT)_n$ (P=0.002)
Negative				
Fukazawa et al. (2005)	Japan	133 MS vs. 156 C	−651C/T −318C/T +49A/G	No association irrespective of *DRB1*1501* status
Bonetti et al. (2004)	Finland	134 simplex families 186 simplex families	−1722 T/C −318 C/T +49 A/G $(AT)_n$	No association
Teutsch et al. (2004)	Australia	129 MS vs. 152 C 97 simplex families	+49 A/G +6230 G/A −1722 T/C −1577 G/A	No association but possible interaction between *+49*G* allele and CD28-*372*G* allele
Bilińska et al. (2004)	Poland	152 MS vs. 154 C	+49 A/G	No association but may influence disease phenotype
van Veen et al. (2003)	Netherlands	514 MS vs. 181 C	−318 C/T +49 A/G	No association
Bocko et al. (2003)	Poland	102 MS vs. 101 C	+49 A/G	No association
Dyment et al. (2002)	Canada	185 multiplex families	+49 A/G $(AT)_n$	No association irrespective of HLA-DRB1*15
Masterman et al. (2002)	Sweden	715 MS vs. 527 C	−318 C/T +49 A/G	No association
Rasmussen et al. (2001)	Denmark China	84 MS vs. 125 C 42 MS vs. 86 C	−318 C/T +49 A/G	No association Possible interaction between CTLA4 haplotype and DR2 in Chinese population only

Abbreviations used: MS—multiple sclerosis, C—healthy controls, PP—primary progressive, RR—relapsing remitting, SP—secondary progressive.

(SNPs), i.e. the well-known +49 A/G and the hitherto not yet investigated CT60 SNP, in susceptibility to MS in a population that has not yet been scrutinized before; i.e. a Belgian–Flemish population. An epidemiological study on MS in Flanders reported a prevalence of 88 per 100,000 in 1991, which is comparable to rates reported in neighbouring areas in Europe around that time (van Ooteghem et al., 1994). Familial risks have been accurately determined: the risk for first-degree and second-degree relatives of MS probands is about 10-fold and 3-fold higher, respectively, than the lifetime risk of the general population (Carton et al., 1997). Among MS patients, 50–55% are positive for the HLA-DR2 allele as compared to only 23–24% among controls (Ghabanbasani et al., 1995; Vandevyver et al., 1994). In this study, we specifically investigated parental transmission of single markers and two-marker haplotypes composed of the +49 A/G and the CT60 SNPs.

2. Subjects and methods

2.1. Subjects

A total of 120 trio MS families (360 individuals) of Belgian–Flemish origin were used in this study. Patients were recruited from the University Hospital Gasthuisberg in Leuven, the National Centre for Multiple Sclerosis in Melsbroek, the University Hospital in Antwerp and the General Hospital Kliniek in Brasschaat. Patients were diagnosed according to the criteria of Poser (Poser et al., 1983) and all the patients had relapsing–remitting MS (RRMS). The patient group consisted of 73 females (60.8%) and 47 males (39.2%) with mean age of onset of disease of 28.0 years (S.D. = ±8.0 years). All the patients and their family members were included in the study after giving written informed consent. Ethics approval for this study was given by the Ethics Committee of the University of Leuven.

2.2. Genotyping

Genotypes of the +49 A/G and CT60 polymorphisms were determined by PCR-restriction fragment length polymorphisms (PCR-RFLP) using *BbvI* and *HypCH4IV* enzymes (New England BioLabs, Beverly, MA), respectively. The +49 A/G polymorphism was amplified with primers 5′-CCA CGG CTT CCT TTC TCG TA-3′ and 5′-AGT CTC ACT CAC CTT TGC AG-3′ (Donner et al., 1997) resulting in a product of 162 bp. The CT60 polymorphism was amplified with primers 5′-AGT GCT TGA TTG CGT GG-3′ and 5′-TGC TGA GAC TAT ACA TTG GTT AAG-3′ (Blomhoff et al., 2004) resulting in a product of 953 bp. The amplified products were digested overnight and analysed on 2% agarose gels. The +49 A/G polymorphism was determined by a 162 bp fragment (representing the A allele) or two fragments of 88 and 74 bp (representing the G allele) while the CT60 polymorphism was determined by a 765 bp fragment (representing the A allele) or two fragments of 614 bp and 151 bp (representing the G allele). The CT60 primers were designed to include a restriction site at 188 bp from the 3′ region to act as an internal control.

2.3. Statistical analysis

The non-transmitted alleles from the parents in the trio families were used as affected family-based controls (AFBAC) according to Thomson (Thomson, 1995). Statistical analysis to compare allelic and genotypic distributions was performed by chi-squared test. The SPSS statistical package (SPSS, Chicago, IL, USA) was used in the analysis of the chi-squared frequencies. Transmission disequilibrium testing (Spielman et al., 1993) was performed using the TRANSMIT (Clayton and Jones, 1999) programme version 2.5, calculating *P* values by means of a bootstrap method (set at 10,000 bootstraps). With 100% genotyping success rate, all family data for both SNPs were complete. TRANSMIT was used for haplotype analysis since it can cope with families with unknown phases. Each SNP was tested for evidence of deviation from Hardy–Weinberg equilibrium using the Arlequin programme version 2.0, which was also used to test for evidence of linkage disequilibrium between polymorphisms. Probability (*P*) values less than or equal to 0.05 were taken to be statistically significant.

Table 2
Allele, phenotype and genotype frequencies of the +49 A/G and CT60 polymorphisms in AFBAC controls and patients

SNP	AFBAC (*N* = 120)	MS patients (*N* = 120)	*P*-value	OR (95% CI)
+49 A/G				
Allele				
A	177 (73.7)[a]	149 (62.1)		
G	63 (26.3)	91 (37.9)	0.006	1.72 (1.16–2.53)
Phenotype				
A	111 (92.5)	104 (86.7)		
G	54 (45.0)	75 (62.5)	0.007	2.04 (1.22–3.41)[b]
Genotype				
AA	66 (55.0)	45 (37.5)		
AG	45 (37.5)	59 (49.2)	0.020	1.92 (1.12–3.31)[c]
GG	9 (7.5)	16 (13.3)		2.61 (1.06–6.42)[d]
CT60				
Allele				
A	119 (49.6)	109 (45.4)		
G	121 (50.4)	131 (54.6)	0.361	1.18 (0.83–1.69)
Phenotype				
A	88 (73.3)	86 (71.7)		
G	89 (74.2)	97 (80.8)	0.216	1.47 (0.8–2.71)[b]
Genotype				
AA	31 (25.8)	23 (19.2)		
AG	57 (47.5)	63 (52.5)	0.462	1.49 (0.78–2.85)[c]
GG	32 (26.7)	34 (28.3)		1.43 (0.69–2.95)[d]

[a]Percentages in parenthesis; Comparison of [b]G, [c]AG and [d]GG carriers vs. AA homozygotes.

3. Results

The +49 A/G polymorphism in the CTLA-4 gene was genotyped in 120 trio MS families with a genotyping success rate of 100% (Table 2). The genotypes of the AFBAC controls were in Hardy–Weinberg equilibrium ($P>0.4$). Comparison of AFBAC and MS +49 A/G allele, phenotype and genotype frequencies revealed statistically significant differences. The phenotype frequency of the G allele was significantly increased in MS patients vs. AFBAC ($P=0.007$ for comparison of G carriers vs. AA homozygotes). The CT60 polymorphism was genotyped in the same set of 120 families (Table 2; 100% success rate). Similarly, the genotypes in the AFBAC controls were in Hardy–Weinberg equilibrium ($P>0.6$). The CT60 allele, phenotype and genotype frequencies did not differ significantly between MS patients and AFBAC.

The results of transmission disequilibrium testing are shown in Table 3. Markers were tested individually and subsequently as a pair. The +49 G allele was significantly over-transmitted to MS patients (TDT statistic=7.860, $P=0.005$). Odds Ratios for G carriers and GG homozygotes, both compared to AA homozygotes, amounted to 2.04 (95% CI 1.22–3.41) and 2.61 (95% CI 1.06–6.42), respectively. There was no significant distortion in the transmission rate of CT60, even though there was a trend towards higher transmission of the G allele to affected children. After performing haplotype analysis, the *+49 A/G*G–CT60*A* haplotype was omitted from the analysis as it was observed at a frequency of less than 5% in the study population. Of the remaining 3 haplotypes, the *+49 A/G*G–CT60*G* haplotype was significantly over-transmitted to affected children (TDT statistic=9.170, $P=0.0025$) while the *+49 A/G*A–CT60*G* haplotype was under-transmitted (TDT statistic=5.880, $P=0.0153$). After correction for testing of 3 major haplotypes, only the association with *+49 A/G*G–CT60*G* remained statistically significant ($P_c=0.0075$). When the three major haplotypes were compared between MS patients and AFBAC controls, the global test of association was highly significant (TDT statistic=12.613, $df=3$, $P=0.0056$).

Table 3
Transmission disequilibrium test for the +49 A/G and CT60 markers and haplotypes[a]

Allele/haplotype	Transmissions	TDT Statistic	*P*-value
*+49 A/G*G*			
Observed	91		
Expected	77	7.860	0.005
*CT60*G*			
Observed	131		
Expected	127	1.633	0.201
*+49 A/G*A–CT60*A*			
Observed	108.0		
Expected	112.0	0.543	0.461
*+49 A/G*A–CT60*G*			
Observed	41.1		
Expected	51.6	5.880	0.0153
*+49 A/G*G–CT60*G*			
Observed	89.9		
Expected	74.4	9.170	0.0025
All 3 major haplotypes	–	12.613	0.0056

[a] *+49A/G*G–CT60*A* excluded as its frequency was less than 5%.

4. Discussion

The CTLA-4 gene has been investigated intensively as a candidate susceptibility gene for a wide variety of autoimmune disorders. With regard to MS, data are conflicting with the majority of reports indicating lack of association with disease (Fukazawa et al., 2005; Bonetti et al., 2004; Teutsch et al., 2004; Biliñska et al., 2004; van Veen et al., 2003; Bocko et al., 2003; Dyment et al., 2002; Masterman et al., 2002; Rasmussen et al., 2001). Prior to this study, a total of 6 *CTLA4* SNPs had been studied in MS, including −1722 T/C, −1577 G/A, −651 C/T, −318 C/T, +49 A/G and +6230 G/A. Of these, the +49 A/G SNP was four times found to be associated with MS in four independent studies, either as single marker, or as part of a three-marker haplotype (Kantarci et al., 2003; Mäurer et al., 2002; Harbo et al., 1999; Ligers et al., 1999). Our study conforms to these findings by revealing parental transmission distortion of the +49 G allele to probands with MS in a population thus far not yet scrutinized, i.e. from the Flanders region in Belgium. In addition, we have for the first time investigated the CT60 polymorphism in MS, and demonstrated that the *+49 A/G*G–CT60*G* haplotype is strongly associated with this disease. Ueda et al. (2003) recently identified the CT60 polymorphism as being the one in the *CTLA4* region most strongly associated with autoimmune disease, and showed the *+49 A/G*G–CT60*G* haplotype to be associated with susceptibility to autoimmunity. Interestingly, this haplotype appeared to be correlated with lower mRNA levels of the soluble alternative splice form of CTLA-4. Though in our study we were not able to demonstrate association of CT60 with MS, our data extend the autoimmune-susceptibility effect of the *+49 A/G*G–CT60*G* haplotype to include MS. Thus, it is likely that the previous findings of association of the G allele of +49 A/G with MS (Kantarci et al., 2003; Mäurer et al., 2002; Harbo et al., 1999; Ligers et al., 1999) may have been due to 'tagging' of this haplotype, and it will therefore be interesting to see whether typing of CT60 in these data sets may validate our findings.

Nevertheless, it remains unclear as to why the majority of studies have not found any indications for *CTLA4* to be involved with MS. Ethnicity or population differences may hardly matter as even in a population from the Netherlands, thought to be closely genetically related to that of Flanders, the +49 A/G SNP was not associated with MS (van Veen et al., 2003). Disease heterogeneity, on the other hand, may affect involvement and nature of genetic susceptibility

factors. On the basis of neurobiological and immunological markers, Lucchinetti et al. (2000) identified four fundamentally different patterns of demyelination in MS. Two of these patterns (I and II) were found to resemble T-cell-mediated or T cell plus antibody-mediated autoimmune encephalomyelitis, while patterns III and IV were characteristic for primary oligodendrocyte dystrophy rather than autoimmunity. The well-documented involvement of CTLA-4 in the regulation of T cell activation may be suggestive for a causative role restricted to patterns I and II. Accordingly, functional genetic effects exerted by haplotypes spanning *CTLA4* may be irrelevant as regards susceptibility to the pattern III and IV form of demyelination in MS. The question, then, arises as to whether the presence or absence of genetic association of *CTLA4* with MS, may be due to inadvertent enrichment—during selection of MS patients for implementation in these genetics studies, for those with expression of a specific subset of demyelination patterns.

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